RESEARCH REPORT

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The effect of microbiome on skin immune system

The human skin surface is colonized by several foreign, apathogenic microorganisms (their community is the human microbiome) which have an essential role in immune mechanisms. Skin microbiome exhibits remarkable variations in distinct topological areas, however the effect of the microbiome heterogeneity on the function of the skin immune system has not been investigated yet. Our aim was to analyse the differences of the immune system on various parts of the normal skin, on dry, sebaceous and apocrine gland rich skin surfaces by monitoring the activity of the skin resident immune cells (keratinocytes, dendritic cells and T cells). These experiments may verify if the skin immune system is unified on the whole body or different skin areas are characterized by distinct skin immune activity.

Biopsy specimens from healthy sebaceous gland poor (SGP), sebaceous gland rich (SGR) and apocrine gland rich (AGR) skin areas were collected, and their immune-cell repertoire, cytokine, chemokine, antimicrobial peptide and barrier molecule expression were investigated both on protein and gene levels. We have supplemented our research with some experiments on immune-mediated skin diseases characteristic to sebaceous gland rich and poor skin areas, and also on apocrine gland rich skin (rosacea, atopic dermatitis, psoriasis and hidradenitis suppurativa).

With our results we proved that the immune system of the normal skin, previously thought to be unified is in fact not unified, but different depending on the skin area. Our research can also provide explanation for the well-known phenomenon, that certain skin diseases occur more often on distinct skin parts like rosacea on the face, psoriasis on extensor while atopic eczemas on flexor surfaces (Griece et al., 2009, Science). Our data should be taken into consideration in future studies aiming to restore healthy skin barrier, or when investigating the pathomechanism of SGR or AGR region-specific inflammatory skin conditions. Moreover, we do not suggest the usage of „healthy skin control" phrase anymore without specifying the region of the sample.

1. **Sebaceous gland rich skin is characterized by TSLP expression and distinct immune surveillance which is disturbed in rosacea**

To examine the effect of microbiome on the immune system of different healthy skin types and to compare the immune milieu of healthy sebaceous gland-rich and sebaceous gland-poor skin areas, biopsy samples were collected from topographically different parts of normal skin (from SGP and SGR skin areas). Moreover, to analyse its changes in an inflammatory disease of SGR skin, samples from rosacea patients (papulopustular rosacea) were also involved in the study. Immunohistochemical, immunocytochemical, and quantitative real-time PCR analyses of thymic stromal lymphopoietin (TSLP) and other cytokines, phenotypic immune cell markers and transcription factors were carried out in the samples. TSLP mRNA and protein production was also studied in cultured keratinocytes.

In SGR skin, higher TSLP expression, dendritic cell appearance without prominent activation, and T cell presence with IL-17/IL-10 cytokine milieu were detected compared with sebaceous gland-poor skin. Linoleic acid, a major sebum component, was found to induce TSLP expression dose-dependently in keratinocytes. In papulopustular rosacea, significantly decreased TSLP level and influx of inflammatory dendritic cells and T cells with IL-17/interferon-γ cytokine milieu were observed. According to our results, SGR skin is characterized by a distinct, noninflammatory immune surveillance, which may explain the preferred localization of inflammatory skin diseases, and can influence future barrier repair therapeutic concepts.

Publication related to the topic:

Zsolt Dajnoki*, Gabriella Béke*, Anikó Kapitány, Gábor Mócsai, Krisztián Gáspár, Ralph Rühl, Zoltán Hendrik, István Juhász, Christos C. Zouboulis, Attila Bácsi, Tamás Bíró, Dániel Törőcsik, Andrea Szegedi. **Sebaceous gland rich skin is characterized by TSLP expression and distinct immune surveillance which is disturbed in rosacea** J Invest Dermatol. 2017 May;137(5):1114-1125. doi: 10.1016/j.jid.2016.12.025. Epub 2017 Jan 25.
*the 2 PhD student equally contributed to this work
(OTKA funding has been properly indicated in the publication)

2. **Topographical Differences in Immune and Barrier Functions of Human Skin**
Since a fine topographical disparity in the activity of the skin immune system was detected by our workgroup, therefore, in our next study, we performed whole transcriptomic and subsequent pathway analyses to further investigate these differences between sebaceous gland rich and sebaceous gland poor regions together with validation of selected molecules at gene and protein levels.

Skin punch biopsies were taken from normal skin of 20 healthy individuals (10 from SGP and 10 from SGR skin sites). In order to explore the in-depth differences between SGR and SGP skin, RNA Sequencing (RNASeq) analysis was performed on whole skin lysates of 6 SGR and 7 SGP patients. The two sample groups could be distinguished unambiguously by the StrandNGS software. Although the differences between the individual SGR and SGP samples on the heat map were not so remarkable as it is often seen in the case of comparing healthy and diseased samples, the principal component analysis (PCA) of the RNASeq data also confirmed that the two healthy groups were highly separated.
With the help of statistical analysis, 1082 genes were found to be differentially (and significantly) expressed in SGR compared with SGP skin; out of these, 672 genes showed higher, whereas 411 genes exhibited lower expressions in the SGR tissues.
Significantly different genes were further analysed by Ingenuity Pathway analysis software (IPA), and they were found to be connected to e.g. lipid and cellular metabolism, cell survival and related pathways, and to IL-17 signalling and IL-17-mediated regulatory functions (both on professional and non-professional immune cells such as macrophages, T cells, and epithelia). These findings were in perfect agreement with our recently published data set, which showed that a significantly higher expression of IL-17A coupled signalling is detected in SGR skin when compared to SGP tissues.

The differential expressions of mRNA transcripts of selected molecules which contribute to skin homeostasis (e.g. molecules of innate and adaptive immunity, cytokines, chemokines and barrier molecules) were confirmed using real-time quantitative PCR (qRT-PCR). We applied this method on an extended number of skin samples (10 SGP and 10 SGR). The selection of these molecules was based on the above RNASeq and IPA analyses, our previously published findings (see e.g. in Dajnoki et al, 2017) related to the IL-17 tissue milieu.

According to our investigations significantly increased chemokine (CCL2, 3, 19, 20, 23, 24) and antimicrobial peptide (S100A7, A8, A9, lipocalin, β-defensin-2) expression, altered barrier (keratin 17, 79) functions and Th17 dominance characterize SGR skin compared to SGP. Regarding proinflammatory molecules (IL-1α, IL-6, IL-8, IL-33, TNF-α), similarly low levels were detected in both regions.

These data should be taken into consideration in future studies aiming to restore healthy SGR skin barrier, or when investigating the pathomechanism of SGR region-specific inflammatory skin conditions. Moreover, we do not suggest the usage of „healthy skin control" phrase anymore without specifying the region of the sample.

Publication related to the topic:

Gabriella Béke†, Zsolt Dajnoki†, Anikó Kapitány, Krisztián Gáspár, Barbara Medgyesi, Szilárd Póliska, Zoltán Hendrik, Zoltán Péter, Dániel Törőcsik, Tamás Bíró, and Andrea Szegedi **Topographical Differences in Immune and Barrier Functions of Human Skin** Frontiers in Immunology (under review)
†the 2 PhD student equally contributed to this work
(OTKA funding has been properly indicated in the publication)

3. **Immune status of healthy apocrine gland-rich skin regions and its alterations in Hidradenitis Suppurativa**

Previously, our workgroup described a non-inflammatory Th17/Treg milieu in sebaceous skin region which can be connected to the different microbiome and chemical milieu of this area. Since these factors also differ in apocrine gland-rich (AGR) areas, we aimed to compare the immune milieu of AGR skin with that of healthy apocrine gland-poor (AGP) skin regions and to study its changes in Hidradenitis Suppurativa (HS), an inflammatory disease characteristically localized on AGR skin. In publications regarding HS the origin of the applied healthy control skin is not consequent; SGP skin is often used instead of AGR skin. Our further aim was to prove, that the correct election of the healthy control skin samples is also very important when investigating the pathomechanism of AGR region-specific inflammatory skin conditions.

Healthy skin biopsy specimens from moist skin areas (axillary region), from apocrine gland poor skin areas and from HS lesional areas were collected, half of them were paraffin-embedded and used for immunohistochemistry, while from the second part of the biopsies the gene expression levels of the above mentioned factors were quantified by qPCR analysis. Cytokines, cell surface markers, activation markers and transcription factors of keratinocytes, dendritic cells (DC) and T cells, and subsequently IL-17 related factors were detected by these methods.

First of all AGR and AGP skin areas were compared to explore if their immune milieu is different, as we observed in SGR and SGP skin. As a results of our IHC experiments, in AGR skin parts higher non-inflammatory TSLP expression, DC appearance without prominent activation and T cell presence with IL-17/IL-10

cytokine milieu were detected compared to AGP skin. Conversely the numbers of these immune cells in moist skin parts were less, than in SGR skin, detected in our earlier projects. No difference was found in the LC counts between the AGR and AGP skin types.

Since we have found a non-inflammatory IL-17 milieu in AGR skin, the effect of IL-17 was also studied by the investigation of IL-17 related chemokines (CCL2, CCL20) antimicrobial peptides (S100A7/8/9, LCN) and barrier elements (FLG, LOR, KRT17). Significantly elevated cytokine, chemokine and AMP expression could be observed in AGR compared to AGP skin, while in the case of barrier proteins no differences could be detected. The qPCR analyses of the aforementioned factors showed similar but less definite pattern.

In HS we strengthened the presence of an inflammatory Th17 milieu, moreover a prominent influx of IL-17+ and IFN-γ+ cells and the presence of activated DCs could be observed in HS when compared to the appropriate control area, to AGR skin. The effect of IL-17 tissue milieu was also studied with the investigation of the aforementioned IL-17 related chemokines, antimicrobial peptides and barrier elements. Significant changes in these factors could be detected, confirming the effect of IL-17 tissue milieu. The qPCR analyses of the aforementioned factors showed a similar but less definite pattern.

Similarly to sebaceous region, AGR skin areas represent distinct immune milieu from AGP (or SGP) regions, which can be the result of the different microbiome and chemical milieu. The aforementioned results may prove that different skin areas are characterized by distinct skin immune activity. Our research may also provide explanation for the well-known phenomenon, that certain skin diseases occur more often on distinct skin parts like rosacea on the face, psoriasis on extensor while atopic eczemas on flexor surfaces.

Publication related to the topic:

Jenei, A, Gaspar, K, Dajnoki, Z, Beke, G, Medgyesi, B, Kinyo, A, Hendrik, Z, Dinya, T, Szegedi, A, Kapitany, A: **Immune status of healthy apocrine gland-rich skin regions and its alterations in Hidradenitis Suppurativa** J Invest Dermatol Volume: 137 Issue: 10 Supplement: 2 Pages: S274-S274 Meeting Abstract: 478 Published: OCT 2017
(OTKA funding is not indicated in the publication)

Adrienn Jenei, Andrea Szegedi, Zsolt Dajnoki, Krisztián Gáspár, Gabriella Béke, Barbara Medgyesi, Ágnes Kinyó, Zoltán Hendrik, Tamás Dinya, Anikó Kapitány:
Immune status and barrier functions of healthy apocrine gland-rich skin regions and its alterations in Hidradenitis Suppurativa (manuscript under preparation)
(OTKA funding will be properly indicated in the publication)

4. **The role of thymic stromal lymphopoietin in the skin and in other barriers**
Thymic stromal lymphopoietin (TSLP) is a critical factor of barriers in sensing the outside word and linking responses. TSLP is expressed predominantly by epithelial cells and, as an important epimmunome molecule, is used to instruct professional immune cells. TSLP is constitutively produced in the thymus and the gut, while in the skin and in the airways, it was detected predominantly in pathological conditions. Mounting evidence has proven that TSLP is also a key player in maintaining immune homeostasis; it is involved in tolerance to commensal microbiota through modulation

of dendritic cell function. Better understanding of TSLP production and its functions will be an important target of future research as it is a potentially important therapeutic target for developing therapies to improve barrier functions. In our review we summarized the current knowledge of TSLP in different barrier surfaces, with the citation of our own results.

Publication related to the topic:

Dajnoki Zsolt, Kapitány Anikó, Béke Gabriella és Szegedi Andrea **The role of thymic stromal lymphopoietin in the skin and in other barriers** Bőrgyógyászati és venerológiai szemle 2017. 93. ÉVF. 3. 119–125. DOI 10.7188/bvsz.2017.93.3.6
(OTKA funding has been properly indicated in the publication)

5. **CD1c+ Blood Dendritic Cells in Atopic Dermatitis are Premature and Can Produce Disease-specific Chemokines.**

We have broadened our research with investigations related to immune-mediated skin diseases localised on SGP skin, like atopic dermatitis (AD) and psoriasis. We have investigated if DC-s separated from the blood of atopic and psoriatic patients bear phenotypically and functionally the same characteristics as their skin counterparts.

Skin dendritic cells of patients with atopic dermatitis are well characterized, but less is known about their peripheral blood precursors. The aim of this study was to investigate the phenotypic features and chemokine production of myeloid pre-dendritic cells of patients with AD ex vivo and after stimulation with Staphylococcus enterotoxin B and thymic stromal lymphopoietin, representing an AD-like microenvironment. The expression of cell surface markers was measured by flow cytometry, while chemokine production was monitored with chemokine antibody array and confirmed by enzyme-linked immunoassays. AD pre-dendritic cells expressed higher levels of FcεRI and the maturation and activation markers tended to be altered. They produced both AD (CCL17/18/22) and maturation-related (CCL3/4/5) chemokines at higher levels than controls. The production of CCL3/4 and CCL18 were significantly higher even without AD-specific stimulation, while the production of CCL17 and CCL22 were significantly higher only after stimulation. These results indicate that circulating AD pre-dendritic cells are premature and bear atopic characteristics even without tissue-specific stimulation, suggesting that their development is not only influenced by the skin microenvironment, but even earlier by the local milieu in the blood.

Publication related to the topic:

Kapitány A, Béke G, Nagy G, Doan-Xuan QM, Bacso Z, Gáspár K, Boros G, Dajnoki Z, Bíró T, Rajnavölgyi É, Szegedi A. **CD1c+ Blood Dendritic Cells in Atopic Dermatitis are Premature and Can Produce Disease-specific Chemokines.** Acta Derm Venereol. 2017 Mar 10;97(3):325-331. doi: 10.2340/00015555-2540.
(OTKA funding has been properly indicated in the publication)

6. **Myeloid but not plasmacytoid blood DCs possess Th1 polarizing and Th1/Th17 recruiting capacity in psoriasis.**

Psoriasis is a common inflammatory skin disease and dendritic cells (DCs) play crucial role in the development of skin inflammation. Although the characteristics of skin DCs in psoriasis are well defined, less is known about their peripheral blood precursors. Our aim was to characterize the phenotypic features as well as the cytokine and chemokine production of $CD1c^+$ myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in the blood samples of psoriatic patients. Blood DCs were isolated by using a magnetic separation kit, and their intracytoplasmic cytokine production and CD83/CD86 maturation/activation marker expression were investigated by 8-colour flow cytometry. In $CD1c^+$ mDCs the intracellular productions of Th1, Th2, Th17, Th22 and Treg polarizing cytokines were examined simultaneously, whereas in pDCs the amounts of IFNα as well as IL-12, IL-23 and IL-6 were investigated. The chemokine production of both DC populations was investigated by flow-cytometry and ELISA. According to our results psoriatic $CD1c^+$ mDCs were in a premature state since their CD83/CD86 maturation/activation marker expression, IL-12 cytokine, CXCL9 and CCL20 chemokine production was significantly higher compared to control cells. On the other hand, blood pDCs neither produced any of the investigated cytokines and chemokines nor expressed CD83/CD86 maturation/activation markers. Our results indicate that in psoriasis not only skin but also blood mDCs perform Th1 polarizing and Th1/Th17 recruiting capacity, while pDCs function only in the skin milieu.

Publication on the topic:

Khasawneh A, Baráth S, Medgyesi B, Béke G, Dajnoki Z, Gáspár K, Jenei A, Pogácsás L, Pázmándi K, Gaál J, Bácsi A, Szegedi A, Kapitány A. **Myeloid but not plasmacytoid blood DCs possess Th1 polarizing and Th1/Th17 recruiting capacity in psoriasis.** Immunol Lett. 2017 Sep;189:109-113. doi: 10.1016/j.imlet.2017.04.005. Epub 2017 Apr 13.

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